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Active compounds present in *Rosmarinus officinalis* leaves and *Scutellaria baicalensis* root evaluated as new therapeutic agents for endometriosis

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Abstract

Research question: Can carnosic acid, (CA) rosmarinic acid (RA) and wogonin (WG) inhibit the growth of cultured human endometrial stromal cells and endometriotic-like lesions induced in a BALB/c model of endometriosis?

Design: Primary stromal cell cultures were established from endometrial biopsies from women with endometriosis and controls. The human endometrial stromal cell line T-HESC was also used for in-vitro experiments. Endometriosis was surgically induced in BALB/c mice, which were randomly assigned to CA 2 mg/kg/day ($n = 11$); CA 20 mg/kg/day ($n = 10$); RA 1 mg/kg/day ($n = 11$); RA 3 mg/kg/day ($n = 10$); WG 20 mg/kg/day ($n = 12$); intraperitoneal vehicle control ($n = 8$) or oral vehicle control ($n = 11$). After surgery, CA and RA were administered intraperitoneally on days 14–28. WG was administered orally by intragastric gavage on days 14–26.

Results: CA, RA and WG significantly inhibited in-vitro cell proliferation in primary and T-HESC cell cultures ($P < 0.05$). CA and WG induced cell cycle arrest of T-HESC at the G2/M phase ($P < 0.01$). RA reduced intracellular ROS accumulation ($P < 0.001$), whereas WG increased it ($P < 0.05$). WG significantly inhibited oestrogen receptor alpha expression in T-HESC ($P < 0.01$). *In-vivo*, CA, RA and WG significantly reduced lesions size ($P < 0.05$). All compounds significantly decreased the percentage of cells in proliferation ($P < 0.05$) whereas RA and WG further increased the percentage of apoptotic cells ($P < 0.05$) in endometriotic-like lesions.

Conclusions: The results are promising; further investigation of these compounds as new therapeutics is needed.

Keywords

Endometriosis; Carnosic acid; Rosmarinic acid; Wogonin

Declaration: The authors report no financial or commercial conflicts of interest.

Introduction

Endometriosis is a chronic disease characterized by the presence of endometrial tissue outside the uterine cavity. This gynaecological disorder affects around 6–10% of reproductive-age women (Viganò *et al.*, 2004), although, in women with infertility, the prevalence increases to 35–50%

(Giudice and Kao, 2004). The most characteristic symptoms of endometriosis are pelvic pain and infertility. Pain, manifested as chronic pelvic pain, dysmenorrhoea and dyspareunia, is frequently intolerable and profoundly affects the quality life of patients and often recurs, even after treatment (Vercellini *et al.*, 2014). Current medical treatments have important disadvantages, including a high rate of disease recurrence and adverse effects that limit their long-term use (Vercellini *et al.*, 2014).

Ectopic endometrial tissue, as well as eutopic tissue, responds to oestrogens and can proliferate and survive outside the uterine cavity. Oestrogen enhances the survival of endometriotic tissue, whereas prostaglandins and cytokines mediate pain, inflammation and infertility (Bulun, 2009; Burney and Giudice, 2012). In addition, chronic inflammation is associated with increased levels of reactive oxygen species (ROS) in endometriosis (Van Langendonck *et al.*, 2002; Ngô *et al.*, 2009; Nasiri *et al.*, 2016).

Although endometriosis is a benign disorder, the process by which endometrial cells attach and invade surfaces shares features of malignancy (Burney and Giudice, 2012). Recent advances in cancer research have focused on the use of extracts and bioactive compounds present in plants (Shanmugam *et al.*, 2011; Ravishankar *et al.*, 2013). It is well known that natural polychemical mixture of pharmacologically active compounds may target multiple vulnerabilities of cancer cells, without toxicity to the non-cancerous cells (Ovadjie *et al.*, 2015). The use of natural compounds provides the possibility of long-term administration with little to no associated toxicity to patients (Shanmugam *et al.*, 2011). The present study, and other studies in endometriosis, also support further investigation of novel, potentially safe and well-tolerated botanical products as innovative therapeutics (Wieser *et al.*, 2012; Ricci *et al.*, 2013; Harlev *et al.*, 2015; Miyashita *et al.*, 2016).

The phenolic diterpene carnosic acid (**Figure 1A**) and the polyphenol rosmarinic acid (**Figure 1B**) are the most abundant active compounds in rosemary (*Rosmarinus officinalis*) leaves and are responsible for the anti-oxidant activity of this plant (Moore *et al.*, 2016). Several studies have recently demonstrated their anti-tumoural (López-Jiménez *et al.*, 2013; Moore *et al.*, 2016) and anti-inflammatory (Yesil-Celiktas *et al.*, 2010; Petiwala and Johnson, 2015) effects.

Similarly, it is known that Chinese herbal medicine is being used for patients with endometriosis and it has had favourable results in decreasing symptoms and increasing fertility (Flower *et al.*, 2012; Stephens *et al.*, 2013; Su *et al.*, 2014; Ried, 2015). On the basis of this evidence, recent studies have aimed to evaluate wogonin (**Figure 1C**), one of the most important bioactive molecules of Chinese herbal medicine. Wogonin is a flavonoid isolated from the root of the medicinal herb Huang-Qin (*Scutellaria baicalensis*). Its anti-tumoural, anti-proliferative, anti-inflammatory and pro-apoptotic properties have already been demonstrated in different studies (Li-Weber, 2009; Wu *et al.*, 2016). Its effects on endometriosis inhibition, however, have not yet been investigated.

Therefore, the aim of our study was to evaluate the effect of carnosic acid, rosmarinic acid and wogonin *in-vitro* and *in-vivo* on the development of experimental endometriosis.

Materials and methods

Patients

A total of 17 reproductive-age patients who underwent diagnostic laparoscopy for infertility participated in this study: eight with untreated endometriosis (Stages II, III and IV, ASRM, 1997), and nine controls. Confirmation of endometriosis was by histological documentation. Control participants included women without endometriosis or any pathology that could affect the evaluated cell population, with tubal factor or unexplained infertility, undergoing diagnostic laparoscopy. All patients were infertile, had regular menstrual cycles and had not received any hormonal medical treatment for the last 6 months. All participants signed informed consent before enrolment. Biopsies of eutopic endometrium were obtained from all participants in the proliferative phase as described previously (Meresman *et al.*, 2000). This study was approved by the Ethics and Research Committee of the IBYME-CONICET on 18 August 2016 (reference CE 11 - Julio/2016).

Isolation and culture of endometrial stromal cells

Endometrial stromal cells were isolated from endometrial biopsies of patients with endometriosis and controls. Stromal cells were enzymatically isolated by successive centrifugations, and primary cultures were established for in-vitro studies. Briefly, tissue was minced, washed and placed in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, Gibco, Fisher Scientific, Loughborough, UK) supplemented with antibiotic–antimycotic (Gibco) and collagenase 0.5 mg/ml (type I, Gibco). After 2 h of incubation at 37°C in a 5% CO₂ atmosphere, the resulting suspension was centrifuged at 400 x g for 5 min. The pellet containing epithelial glands and stromal cells was resuspended in culture medium and filtered through a 40 µm cell strainer (BD FalconTM, USA). The solution containing mainly stromal cells was recovered and centrifuged at 400 x g for 5 min. Finally, stromal cells were counted and plated with DMEM/F12

supplemented with 10% fetal bovine serum (FBS) (Gibco) in a humidified environment with 5% CO₂ at 37°C. Purity of the culture was determined by vimentin immunostaining, resulting in a 90% stromal-cell culture (data not shown).

Cell line and culture conditions

Telomerase-immortalized Human Endometrial Stromal Cell line (T-HESC, ATCC CRL-4003, USA) was cultured in 10% FBS DMEM/F-12 in a humidified 5% CO₂ incubator at 37°C.

Cell proliferation assay

Five thousand T-HESC cells per well and 1.5×10^4 endometrial stromal cells per well from patients and controls were individually plated in 96-well culture plates with 10% FBS DMEM/F12 and incubated at 37°C in a 5% CO₂ atmosphere. When cells reached 70% confluence, cultures were washed and incubated for 24 h with different concentrations of each compound in fresh 1% FBS medium. The evaluated treatments were: 2.5, 5, 7.5, 10, 12.5 and 25 µg/ml of carnosic acid (Alexis biochemicals, San Diego, CA, USA); 25, 50 and 100 µg/ml of rosmarinic acid (Enzo Life Sciences, Farmingdale, NY, USA) and 40, 80 and 160 µM of wogonin (Sigma-AldrichTM, St Louis, MO, USA). Basal conditions were obtained by incubating cells with the vehicles used to dissolve each compound: dimethyl sulphoxide (DMSO) (Sigma-Aldrich) at a final concentration less than 0.8% for carnosic acid and wogonin, and phosphate buffered saline (PBS) for rosmarinic acid. Each treatment condition was run in quadruplicate. Cell viability was determined by a colorimetric assay using the Cell titer 96 Aqueous One

Solution Cell proliferation kit ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], MTS, Promega Corp., Fitchburg WI, USA) as previously described (Ricci *et al.*, 2013).

Cell cycle analysis

For cell cycle analysis 2.5×10^5 T-HESC cells per well were plated in 6-well culture plates with 10% FBS DMEM/F12. After 24 h, cultures were washed and incubated for an additional 48 h with carnosic acid (0, 2.5 and 5 $\mu\text{g/ml}$), rosmarinic acid (0, 50 and 100 $\mu\text{g/ml}$) or wogonin (0, 40 and 80 μM) in 1% FBS-supplemented medium. Next, the cells were harvested using trypsin 0.25% (Gibco) and centrifuged at $250 \times g$ for 5 min. Supernatant was removed, cells were washed with ice-cold PBS, and fixed with ice-cold 70% ethanol. Cells were kept at -20°C until assayed. Then, cells were centrifuged at $100 \times g$ for 5 min and the supernatant was removed. The cell pellet was carefully resuspended adding 50 $\mu\text{g/ml}$ propidium iodide in PBS. Cells were kept in the dark for 15 min and cell cycle distribution was determined using a flow cytometer (FACS Canto II, BD Biosciences, San Jose, CA, USA). Cyflogic1.2.1 software was used to analyse the results. All treatments were compared with control condition incubated with vehicle.

Determination of intracellular reactive oxygen species levels

2',7'-Dichlorodihydrofluorescein diacetate ($\text{H}_2\text{DCF-DA}$) (Sigma-Aldrich) was used to assess the intracellular ROS levels (Rossi *et al.*, 2016). After penetration into the cells, the non-fluorescent ester was hydrolyzed to 2',7'-dichlorodihydrofluorescein (H_2DCF) by cellular esterases. H_2DCF was oxidized to the highly fluorescent compound 2',7'-dichlorofluorescein (DCF) by

intracellular ROS. Six thousand T-HESC cells per well were plated in 96-well black/clear bottom culture plates. After a 24-h treatment with carnosic acid (0; 2.5; 5; 7.5 and 10 $\mu\text{g/ml}$), rosmarinic acid (0; 50 and 100 $\mu\text{g/ml}$) or wogonin (0; 40 and 80 μM), cells were incubated in a medium containing 10 μM $\text{H}_2\text{DCF-DA}$ for 30 min in the dark at 37°C and 5% CO_2 . Dichlorofluorescein (generation was measured every 5 min for 70 min at 37°C using a spectrofluorometer (Molecular Devices Spectramax Gemini EM) at 485 nm excitation and 530 nm emission. The Δ fluorescence was calculated by subtracting the average fluorescence intensity at 0 min to the average fluorescence intensity at 70 min. To include an anti-oxidant control, cells were pre-treated with 5 mM of N-acetyl-cysteine, a ROS scavenger, for 1 h and then the highest dose of wogonin was added.

Western blot

A total of 2.5×10^5 T-HESC cells per well were plated in 6-well culture plates with 10% FBS DMEM/F12. After 24 h, cultures were washed and incubated for an additional 48 h with carnosic acid (0, 2.5 and 5 $\mu\text{g/ml}$), rosmarinic acid (0, 50 and 100 $\mu\text{g/ml}$) or wogonin (0, 40 and 80 μM) in 1% FBS-supplemented medium. Cells were then lysed by scraping in chilled lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 10% glycerol, 1% NP40, 1mM MgCl_2 , 0.1% SDS) supplemented with protease inhibitors (1:500, Sigma). After freeze thawing, the lysate was centrifuged at 15,000 $\times g$ for 10 min at 4°C, and the pellet was discarded. Protein concentrations in the supernatant were measured by the Bradford assay (Bradford, 1976). Fifty μg of proteins were separated from each sample and subjected to a 12% polyacrylamide gel electrophoresis for about 90 min at 150 V. The separated proteins were transferred to a 2 μm nitrocellulose membrane (Hybond ECL, Amersham Biosciences), which was blocked for 1 h in 5% low-fat powdered milk at room temperature. Then, it was incubated overnight with the

oestrogen receptor alpha primary antibody (1:250, clone MC-20, Santa Cruz Biotechnology, Dallas, TX, USA) or glyceraldehyde 3-phosphate dehydrogenase antibody (1:10000, clone 14C10, cell signalling) diluted in 1% low-fat powdered milk in 0.01% tween in tris buffer saline at 4°C, followed by the peroxidase-conjugated secondary antibody (anti-rabbit IgG, 1:2000, Sigma) diluted in tween in tris buffer saline at room temperature for 1 h. Protein bands were visualized by chemiluminescence, incubating the membranes for 1 min with the ECL chemiluminescent reagent (Pierce). The images of the bands were digitally acquired (G-Box, SynGene). The quantification was carried out by densitometric analysis of the bands, using the Image J software (NIH), normalized with the glyceraldehyde 3-phosphate dehydrogenase expression in each well.

Measurement of oestradiol by enzyme-linked immunosorbent assay

Oestradiol levels were measured in conditioned media from T-HESC cell cultures treated for 48 h with wogonin (0, 40, 80 μ M) using a commercial enzyme-linked immunosorbent assay (ELISA) kit and following the procedures detailed by the manufacturer (Estradiol ELISA kit, Cayman Chemical, Ann Arbor, MI, USA). The conditioned media was centrifuged (5 min, 100 x g) and the supernatants were maintained at -20°C. Before the test, steroid hormones were extracted using ethyl ether. Briefly, the conditioned media was shaken for 1 min in conical glass tubes after adding ethyl ether in a 1:2 ratio and incubated at -70°C for 1 h. Then, the ether phase was transferred to another tube and was evaporated in a thermal bath at 50°C. Ethyl ether was added

again to the initial tube and the steps mentioned above were repeated. Once the evaporation of the ether was completed, the extracts were stored at -70°C until the day on which the ELISA was carried out. The oestradiol quantification was carried out according to procedures detailed by the manufacturer. The sensitivity level for the oestradiol ELISA was 15 pg/ml. A spectrophotometer (Labsystems Multiscan, Midland, ON, Canada) was used to record absorbance at 405 nm. All samples were assessed in duplicate.

Animals

In this study, 77 2-month-old female BALB/c mice, weighing 20–24 g were used. All procedures were carried out according to the National Institutes of Health *Guide for the care and use of laboratory animals* and approved by the CICUAL Committee from the IBYME-CONICET on 18 August 2016 (reference CE 11 - Julio/2016). A total of four animals died or were killed 2 or 3 days after surgery because they did not fully recover from the procedure.

All animals were weighed every other day so that they received the correct dose of the corresponding treatment. This measurement also served as a control for their overall wellbeing. Additionally, animals were carefully observed to detect any changes in their grooming behaviour, activity levels and food consumption from post-surgical day 1 up to day of sacrifice.

During treatment, vaginal smears were obtained to check oestrous cycle progression. A total of 20 μl saline solution was introduced to the vaginal canal and the withdrawn solution was mounted on a slide and observed microscopically for cytological staging. This procedure was continued over 8 days.

Surgical induction of endometriosis and treatment

Endometriosis-like lesions were induced through transplantation of one of the uterine horns to the bowel mesentery as previously described (Olivares *et al.*, 2011). All treatments started on postoperative day 14, at which point the endometriotic lesions were already developed (Nisolle *et al.*, 2000). Carnosic acid and rosmarinic acid were administered intraperitoneally over 14 consecutive days; wogonin was administered orally by intragastric gavage over 12 consecutive days.

Carnosic acid (Enzo Life Sciences, USA) was dissolved in dimethyl sulfoxide (DMSO) and diluted in physiological solution (0.8% DMSO final concentration). Rosmarinic acid (Enzo Life Sciences) was dissolved in PBS. Both treatments shared the control group, which received vehicle (control intraperitoneal 0.8% DMSO in physiological solution). Wogonin (Enzo Life Sciences) was partially resuspended in DMSO and diluted in physiological solution for its administration (1.6% DMSO final concentration); its control group received vehicle by the same route of administration (control oral). Treatment groups, doses and routes of administration are presented in **Table 1**.

Because of the lack of research on the bioavailability of these compounds after oral administration, carnosic acid and rosmarinic acid were administered intraperitoneally to observe a local and direct effect. Wogonin was administered orally because it is difficult to dissolve.

Endometriotic like-lesions evaluation

Animals were sacrificed by cervical dislocation 24 h after the last injection. The abdomen was opened by ventral midline incision. Implantation sites were localized by the presence of a lesion or by suture alone. Lesions were counted and measured for volume determination using the formula: $V = (4/3)\pi r^2 R$ (where r and R are the radiuses, $r < R$) (Brodie *et al.*, 2003). Then, lesions were excised and fixed in 4% formaldehyde, paraffin-embedded and cut into 5- μ m serial sections for histological analysis. Several sections from each specimen were stained with haematoxylin and eosin, and examined microscopically for the presence of histological hallmarks (glands and stroma) of endometriosis.

Immunohistochemistry for proliferating cell nuclear antigen

Serial sections of endometriotic-like lesions were subjected to standard immunohistochemistry procedures for proliferating cell nuclear antigen (PCNA) as described previously (Olivares *et al.*, 2011) using rabbit anti-mouse PCNA polyclonal (1:800, FL-261, Santa Cruz Biotechnology, Santa Cruz, USA). The presence of brown nuclear reactivity indicated PCNA-positive cells. The above was carried out in negative controls except that the primary antibody was replaced with a rabbit immunoglobulin G isotype antibody (1:800, ab37415, Abcam, Cambridge, UK). The number of cells immunopositive for PCNA was established using a standard light microscope at 400 X magnification. At least 400 epithelial and stromal cells from representative fields were counted by two independent observers blinded to the treatment condition, and the percentage of PCNA-positive cells was calculated.

TUNEL assay

For apoptosis quantification, sections were processed for TdT (terminal deoxynucleotidyl transferase) mediated dUDP nick-end labelling (TUNEL) staining using the In Situ Cell Death POD kit (Roche, Penzberg, Germany). Sections were treated according to the manufacturer's instructions (Bilotas *et al.*, 2015). The number of TUNEL positive cells was established using a standard light microscope at 400 X magnification. At least 400 epithelial and stromal cells from representative fields were counted by two independent observers blinded to the treatment condition, and the percentage of TUNEL positive cells was calculated.

Statistical analysis

GraphPad PRISM software V4.0 (GraphPad Software Inc, La Jolla, CA, USA) was used for statistical analysis. Statistical comparisons between groups were carried out using parametric one-way analysis of variance followed by Tukey's multiple comparison test. Student t-test was used for statistical comparisons between two groups, with Welch correction in groups with significantly different variances. Results were expressed as mean \pm SEM. In all cases statistical significance was considered when $P < 0.05$.

Results

In-vitro results

Effect of carnosic acid, rosmarinic acid and wogonin on cell proliferation

The effect of different concentrations of each natural compound on cell proliferation of primary cultures of endometrial stromal cells and on cultures of the T-HESC cell line are presented in **Figure 2**. After exposure to 10 μ g/ml of carnosic acid, endometrial stromal cells from control and

endometriosis patients showed significantly lower percentage of cell proliferation than basal conditions ($P < 0.001$ and $P < 0.05$, respectively, versus basal) (**Figure 2A i-ii**). Similar results were observed with the stromal cell line, where 10, 12.5 and 25 $\mu\text{g/ml}$ of carnosic acid reduced the percentage of cell proliferation ($P < 0.05$, $P < 0.001$ and $P < 0.001$ respectively, versus basal) (**Figure 2A iii**).

The highest concentrations of rosmarinic acid, 50 and 100 $\mu\text{g/ml}$, reduced cell proliferation in cultures from control women ($P < 0.05$ and $P < 0.01$ respectively, versus basal) (**Figure 2B i**), but only rosmarinic acid 100 $\mu\text{g/ml}$ showed a significant effect in primary cell cultures from endometriosis patients ($P < 0.05$ versus basal) (**Figure 2B ii**). Similar to the results observed in primary cultures, rosmarinic acid 50 and 100 $\mu\text{g/ml}$ showed a significant inhibition of T-HESC proliferation in a dose-dependent manner ($P < 0.05$ and $P < 0.001$, respectively versus basal) (**Figure 2B iii**).

In the same way, wogonin 80 μM caused a reduction in the percentage of cell proliferation in cultures from control women ($P < 0.001$ versus basal) (**Figure 2C i**) and wogonin 40 and 80 μM induced cell proliferation inhibition in cultures from endometriosis patients ($P < 0.05$ versus basal) (**Figure 2C ii**). In addition, wogonin 40, 80 and 160 μM caused a reduction on cell proliferation of T-HESC ($P < 0.05$, $P < 0.001$ and $P < 0.001$ respectively, versus basal) (**Figure 2C iii**).

These results demonstrate the effectiveness of these natural compounds on the diminution of the percentage of cell proliferation. So far, as the results obtained for primary endometrial stromal

cell cultures and the T-HESC cell line were similar, we decided to continue the analysis only with the cell line.

Effect of carnosic acid, rosmarinic acid and wogonin on cell cycle progression

The mean per cent distribution of T-HESC in the different phases of the cell cycle is shown in **Figure 3**. G0/G1 phase decreased significantly after incubation with 2.5 and 5 $\mu\text{g/ml}$ of carnosic acid ($P < 0.01$ and $P < 0.001$ versus control, respectively); 5 $\mu\text{g/ml}$ of carnosic acid increased the DNA synthesis (S) phase ($P < 0.05$ versus control) and the percentage of cells in the G2/M phase was also significantly increased with both concentrations of this compound ($P < 0.01$ and $P < 0.001$ versus control, respectively) (**Figure 3A**). It should be highlighted, that the concentrations of carnosic acid tested for cell cycle were lower than those that had an effect on cell proliferation. This is because, for this assay, the cells were stimulated during 48 h instead of 24 h, and higher concentrations provoked detaching of the cells and the analysis could not be conducted.

After incubation with 50 and 100 $\mu\text{g/ml}$ of rosmarinic acid, G0/G1 and G2/M phases of the cell cycle distribution of T-HESC were not altered (**Figure 3B**). A significant decrease in the S phase was induced with 50 $\mu\text{g/ml}$ of rosmarinic acid compared with basal condition (**Figure 3B**).

Similar to the results obtained with carnosic acid, G0/G1 phase decreased significantly after incubation with wogonin 40 and 80 μM ($P < 0.001$ versus control). In addition, wogonin 40 μM increased significantly the S phase ($P < 0.05$ versus control) and the percentage of cells in the G2/M phase was also significantly increased with wogonin 40 and 80 μM ($P < 0.001$ versus control) (**Figure 3C**).

In summary, cell cycle analysis monitored by flow cytometry revealed that carnosic acid and wogonin induced cell cycle arrest at the G2/M phase. These results suggest that inhibition of endometrial stromal cells proliferation after treatment with these two compounds might be associated with cell cycle arrest induction.

Effect of carnosic acid, rosmarinic acid and wogonin on intracellular reactive oxygen species production

Carnosic acid did not alter the intracellular ROS levels (**Figure 4A**); however, rosmarinic acid 50 and 100 µg/ml induced a significant decrease of fluorescence intensity, suggesting a strong anti-oxidant power of this compound ($P < 0.001$ versus control) (**Figure 4B**). Conversely, after wogonin 80 µM treatment, Δ fluorescence was increased compared with untreated cells ($P < 0.05$, (**Figure 4C**). Pre-treatment with N-acetyl-cysteine slightly reduced wogonin-generated ROS to control levels. This result indicates that wogonin 80 µM had a pro-oxidant effect on T-HESC.

Effect of carnosic acid, rosmarinic acid and wogonin on oestrogen receptor alpha expression and oestradiol secretion

After 48 h treatment with the rosemary bioactive compounds carnosic acid and rosmarinic acid, oestrogen receptor alpha expression was slightly decreased but the differences were not statistically significant compared with the control group (**Figure 5A–5B**). On the other hand, wogonin 40 and 80 µM induced a significant decrease of oestrogen receptor alpha expression ($P < 0.01$ and $P < 0.001$, respectively) versus controls (**Figure 5C**). This result could be associated with wogonin anti-proliferative effect observed in T-HESC cell line. No differences, however, were observed on oestradiol secretion by T-HESC after wogonin treatment (**Figure 6**).

In-vivo results

No evidence of toxicity was found at the doses administered based on body weight (controlled every other day), food consumption, grooming behaviour, or activity levels compared with controls. Oestrous cycles were followed cytologically during treatments. No differences were detected between controls and treated mice, which suggests that treatments did not interfere with normal oestrous cycle progression (data not shown).

Effect of carnosic acid, rosmarinic acid and wogonin on endometriotic-like lesion growth

The highest doses of all treatments caused a statistically significant reduction in lesion size developed in mice. Administration of 20 mg/kg/day of carnosic acid, 3 mg/kg/day of rosmarinic acid and 20 mg/kg/day of wogonin diminished the volume of developed lesions ($P < 0.01$; $P < 0.05$; $P < 0.05$ versus controls, respectively) (**Figure 7 Ai–Ci**). The number of established lesions observed after treatment, however, was similar to controls (**Figure 7 Aii–Cii**).

Effect of carnosic acid, rosmarinic acid and wogonin on cell proliferation in endometriotic-like lesions

Cell proliferation was evaluated by immunohistochemistry for PCNA (**Figure 8**). Results were expressed as the percentage of PCNA positive cells. As it can be seen in **Figure 8 Ai–Ci**, all treatments significantly decreased cell proliferation in endometriotic-like lesions compared with the control group (carnosic acid and wogonin: $P < 0.05$, rosmarinic acid: $P < 0.001$). Representative micrographs of PCNA immunostaining are shown in **Figure 8 Aii–Cii**.

Effect of carnosic acid, rosmarinic acid and wogonin on apoptosis in endometriotic-like lesions

The effect of the natural compounds on apoptosis are presented in **Figure 9**. After carnosic acid treatment, a slight increase in the percentage of TUNEL positive cells in endometriotic-like lesions was observed, but the differences were not statistically significant compared with the control group (**Figure 9A i**). On the other hand, the highest dose of rosmarinic acid (3 mg/kg/day) significantly increased the percentage of TUNEL-positive cells in endometriotic-like lesions ($P < 0.05$ versus control group) (**Figure 9B i**). In the same way, after treatment with wogonin (20 mg/kg/day) the percentage of TUNEL positive cells was significantly higher than the control group ($P < 0.001$) (**Figure 9 Ci**). Representative micrographs of TUNEL technique are shown in **Figure 9 Aii–Cii**.

Discussion

Current medical treatments available for endometriosis are not curative *per se* but they are nonetheless helpful to alleviate symptoms and prevent recurrence after surgery. Standard treatments for endometriosis involves laparoscopic ablation of endometriotic lesions and, in some cases, suppression of ovarian function with hormonal drugs, which also contribute to reduce the pain associated with endometriosis (Muñoz-Hernando *et al.*, 2015). Additionally, pain management options include non-steroidal anti-inflammatory drugs and analgesics. Significant adverse effects are known when these drugs are used for a long period of time (Quaas *et al.*, 2015). Considering the disadvantages and limitations that conventional treatments carry, we focused recent efforts on looking for new natural treatments with the intention of achieving high

efficiency, avoiding adverse effects and, as a desirable ultimate goal, preserving the chances of conceiving a successful pregnancy.

Our in-vitro results demonstrated the effectiveness of carnosic acid and rosmarinic acid on inhibiting endometrial stromal cell proliferation and, in particular, on inducing G2/M cell cycle arrest by carnosic acid. These results are in accordance with those of Visanji *et al.* (2006) who demonstrated that the anti-proliferative effect of carnosic acid on human epithelial colorectal adenocarcinoma cells is associated with alterations of Cyclin A and Cyclin B1 levels. These proteins are responsible for cell cycle progression and a deregulated expression could cause cell cycle arrest predominantly at G2/M phase. In addition, Yesil-Celiktas *et al.* (2010) demonstrated significant anti-proliferative activity of carnosic acid in various human cancer cell lines. Deregulation of cell cycle and pro-apoptotic properties of these compounds have also been variously reported (Tai *et al.*, 2012; López-Jiménez *et al.*, 2013; Min *et al.*, 2014). Nevertheless, their effects on endometriosis inhibition had not been investigated until now.

Given the importance of oestrogen in this pathology, we decided to evaluate whether oestrogen receptor alpha expression was altered after treatment, but neither carnosic acid nor rosmarinic acid had an effect on the expression of this receptor. According to this result, we cannot account the inhibition of proliferation to be due to this pathway. A similar observation was made by Einbond *et al.* (2012) who demonstrated that carnosic acid inhibited the cell proliferation of an oestrogen receptor alpha-negative breast cancer cell line (Einbond *et al.*, 2012).

Moreover, a strong anti-oxidant effect was observed after the stimulus with rosmarinic acid in T-HESC cells, suggesting a possible role for ROS in the regulation of cell proliferation. Our results

are in accordance with other investigators who had also confirmed the anti-oxidant property of rosemary. Cattaneo *et al.* (2015) have demonstrated an anti-oxidant effect of rosemary extract on melanoma cells. Similarly, it was discovered that rosmarinic acid was able to decrease ROS levels in endothelial cells and in a colon cancer cell line using concentrations ranging from 9 to 100 µg/ml (Huang and Zheng, 2006; Xu *et al.*, 2010).

Endometriosis is associated with chronic inflammation and ROS increase; therefore, these results are encouraging as the anti-oxidant properties of polyphenols provide anti-inflammatory and chemopreventive abilities (Ravishankar *et al.*, 2013; Stepanic *et al.*, 2015).

In addition to studies demonstrating the in-vitro effects of rosemary, several studies using animal models have also provided strong evidence for its anti-tumoural effect. A recently published review summarized the in-vivo anti-cancer properties of carnosic acid and rosmarinic acid (Moore *et al.*, 2016). Studies supporting tumour growth inhibition have been conducted in animal models of prostate cancer (Petiwalla *et al.*, 2013; 2014) and colorectal cancer (Ngo *et al.*, 2011) among others. Similar to the results found in our in-vivo experiments, it was reported that oral administration of 100 mg/kg of rosemary extract for 22 days decreased the prostate cancer xenograft tumour size in 46% of athymic nude mice (Petiwalla *et al.*, 2014). In addition, intraperitoneal administration of 2 mg/kg of rosmarinic acid for 20 days significantly reduced the weight of solid lung carcinoma tumours developed in C57BL/6 mice as well as it effectively inhibited tumour metastasis in this model (Xu *et al.*, 2010).

The results obtained in our study using an in-vivo mouse model of endometriosis are congruent with the investigations cited above. Our results showed that carnosic acid and rosmarinic acid

were effective in reducing the size of endometriotic-like lesions'. We observed a significant inhibition on cell proliferation after treatment with both polyphenols and apoptosis induction after treatment with rosmarinic acid, in endometriotic-like lesions developed in mice.

Traditional Chinese medicine is prescribed in China for the treatment of a variety of inflammatory diseases, including endometriosis. *Scutellaria baicalensis* is one of the fundamental herbs present in Chinese medicine (Li-Weber, 2009; Lee *et al.*, 2014), and wogonin is the main flavonoid isolated from the root of this plant. Our results provide evidence of an anti-proliferative effect of wogonin on human endometrial stromal cells added to cell cycle arrest induction at G2/M phase.

Similarly, inhibition of cell proliferation, cell cycle arrest and apoptosis induction are some of the mechanisms that have been demonstrated to be altered after wogonin treatment in several cancer cell lines (Yu and Kim, 2011; Tao *et al.*, 2014). Tao *et al.* (2014) reported that LL-202, a newly synthesized flavonoid derived from wogonin, induced arrest in cell cycle progression at G2/M phase in the breast cancer cell line MCF-7, which is in line with our results. More recently, Kavandi *et al.* (2015) demonstrated that *Scutellaria baicalensis* extract is able to inhibit proliferation of ovarian and endometrial cancer cell lines through inhibition of NF κ B activation.

Although flavonoids are commonly known for their anti-oxidant activity (Ravishankar *et al.*, 2013; Stepanic *et al.*, 2015), many investigators have also demonstrated their pro-oxidant properties (Galati and O'Brien, 2004; Lin *et al.*, 2008; Tao *et al.*, 2014). This property can be useful in producing cytostatic effects specifically on cancer cells. The selectivity is due to the fact that cancer cells, compared with normal cells, intrinsically increase ROS, partly as a result of

oncogenic stimulation, increased metabolic activity and mitochondrial malfunction (Hileman *et al.*, 2004; Pelicano *et al.*, 2004). As the endometrial cells at the ectopic site behave in a similar way to neoplastic cells, it is not surprising that treatments have similar effects. Our results provide evidence that wogonin promoted the intracellular ROS accumulation in T-HESC cells. We suggest that this pro-oxidant activity could induce cell death by apoptosis, a mechanism that has already been reported by other authors (Baumann *et al.*, 2008; He *et al.*, 2012; Yu and Kim, 2011). Yu and Kim (2011) have shown that wogonin-induced apoptosis in human breast cancer cells is coupled with the generation of ROS and activation of ERK and p38. In the same way, He *et al.* (2012) have demonstrated that wogonin 10 μ M was able to induce apoptosis of two cell lines, A549 and HeLa (pulmonary tumour cells and cervical cancer cells, respectively) through the intracellular accumulation of H_2O_2 .

Although wogonin decreased oestrogen receptor alpha expression in T-HESC cells, oestradiol secretion was not altered. This suggests that cell proliferation inhibition would not be mediated only through this pathway. In agreement, Chung *et al.* (2008) observed that the inhibition of cell proliferation induced by wogonin occurred both in oestrogen receptor alpha positive and negative mammary tumour cells, concluding that the inhibition of oestrogen receptor alpha expression would only partially contribute to the reduction of proliferation of positive oestrogen receptor alpha cells (Chung *et al.*, 2008).

Furthermore, we demonstrated the in-vivo effectiveness of this flavonoid on reduction of endometriotic-like lesions size and an anti-proliferative and pro-apoptotic effect of wogonin within the lesions. Studies published in the last few years prove the anti-tumour effect of wogonin in animal models (Wu *et al.*, 2016). Baumann *et al.* (2008) have demonstrated that

wogonin was able to suppress growth of human T-cell leukaemia xenografts in immunodeficient mice (Baumann *et al.*, 2008). In the same way, the in-vivo effect of oral wogonin administration was examined on human breast cancer xenografts growth in athymic nude mice. Treated mice showed inhibition of tumour growth by up to 88% without any toxicity after treatment (Chung *et al.*, 2008). In BALB/c nude mice, growth of xenotransplanted MCF-7 tumours was also inhibited after 21 days of treatment with LW-213, a chemically synthesized flavonoid derived from wogonin. LW-213 inhibited cell proliferation in tumors and altered cell cycle regulatory proteins (Zhao *et al.*, 2016).

In conclusion, reported mechanisms by which the studied compounds exert their action are complex and variable. Cited studies definitely reveal the versatility of natural compounds in targeting multiple cell pathways, all of these especially important in cancer (Ovadjie *et al.*, 2015) and hence in endometriosis pathophysiology.

To the best of our knowledge, this is the first study evaluating the effects of carnosic acid, rosmarinic acid and wogonin on the growth of human endometrial stromal cells and on the development of endometriotic-like lesions in a rodent model (**Figure 10**). These results are promising and lead to several open research questions that we are only beginning to explore. Better understanding of the basic mechanisms of action of these natural compounds, still unknown in endometrial cells, is needed to determine the potential usefulness of these natural compounds as endometriosis preventive or therapeutic agents.

Acknowledgements

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Author biography



Gabriela Meresman, PhD, is Head of the Laboratory of Endometrial Pathophysiology at the Institute of Experimental Biology and Medicine in Argentina. Her research interests include the discovery of new targets for endometriosis treatment and evaluating natural compounds that could act directly on the implants as an alternative to current treatments.

Key message

Current medical treatments available for endometriosis have drawbacks. Carnosic acid, rosmarinic acid and wogonin have an inhibitory effect on in-vitro stromal endometrial growth and in-vivo endometriotic-like lesions development. The findings are promising and support further investigation of these novel, potentially safe and well-tolerated botanical products as future endometriosis treatments.

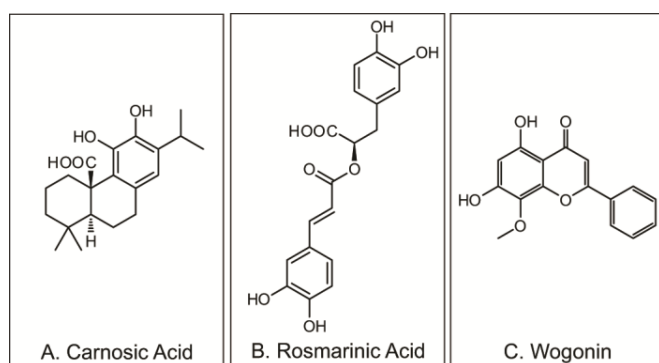


Figure 1. Natural compounds evaluated as new therapeutic agents for endometriosis.

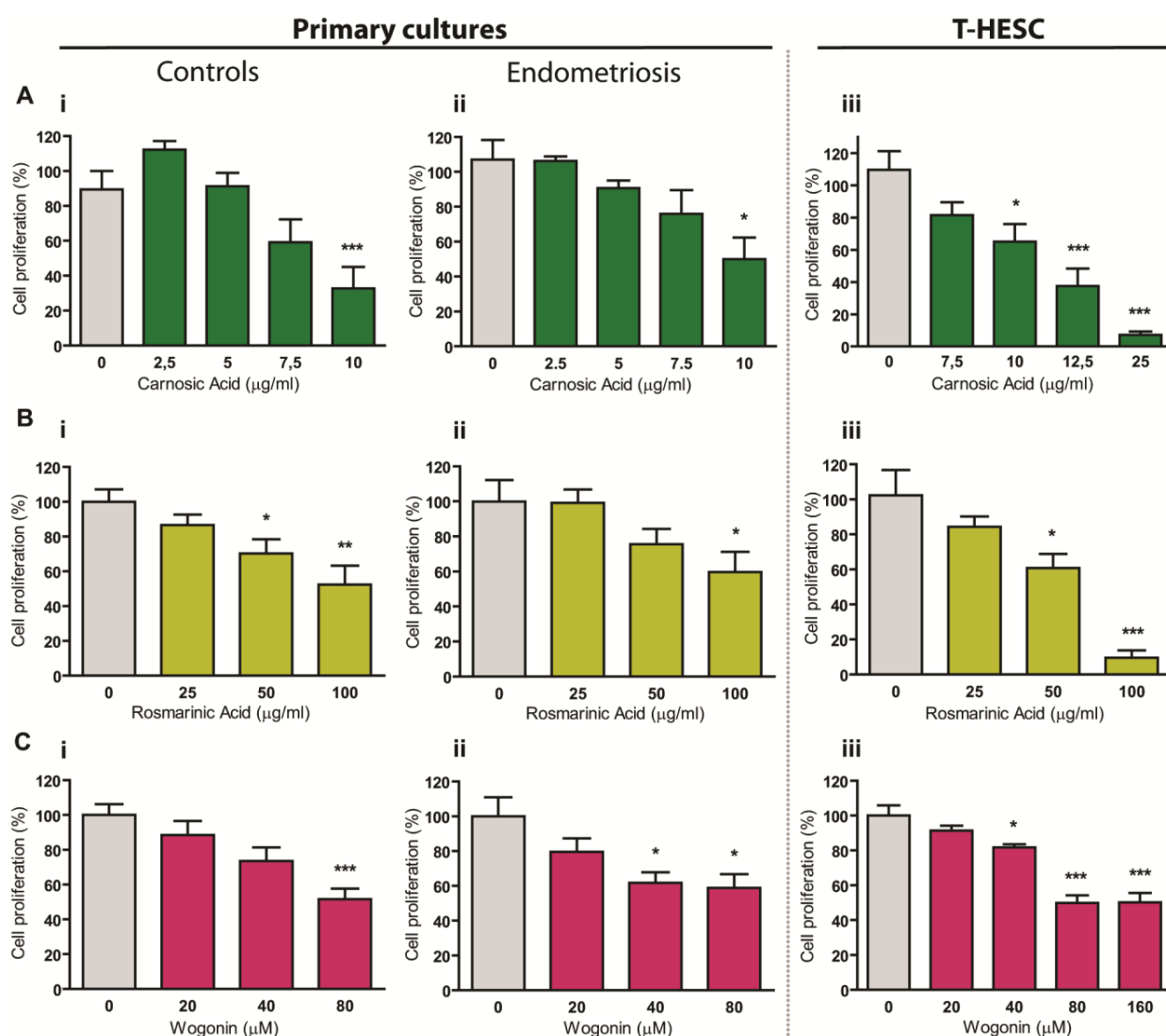


Figure 2. Effect of carnosic acid, rosmarinic acid and wogonin on cell proliferation of primary cultures of endometrial stromal cells and T-HESC cell line. Cell proliferation was assessed by MTS assay. (A) Carnosic acid; (B) rosmarinic acid; (C) wogonin. (i) Endometrial stromal cells from control women ($n = 7$); (ii) endometrial stromal cells from patients with endometriosis ($n = 7$); (iii) T-HESC ($n = 5$). Values of each treatment condition are expressed as a percentage of basal cell proliferation (set as 100%). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus basal.

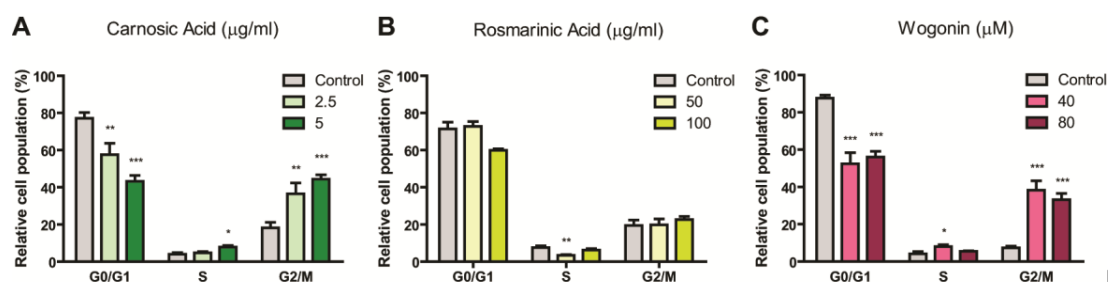


Figure 3. Effect of carnosic acid, rosmarinic acid and wogonin on cell cycle progression. Cell cycle was monitored by flow cytometry staining the DNA with propidium iodide. (A) Carnosic acid ($n = 7$); (B) rosmarinic acid ($n = 7$); (C) wogonin ($n = 6$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus control.

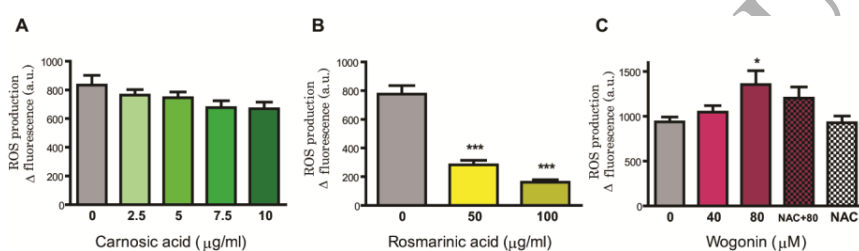


Figure 4. Effect of carnosic acid, rosmarinic acid and wogonin on intracellular reactive oxygen species (ROS) production in treated T-HESC cells detected by using $\text{H}_2\text{DCF-DA}$ measured with a spectrofluorometer. (A) Carnosic acid ($n = 7$); (B) rosmarinic acid ($n = 6$); (C) wogonin ($n = 9$). * $P < 0.05$; ** $P < 0.001$ versus control. au, arbitrary units.

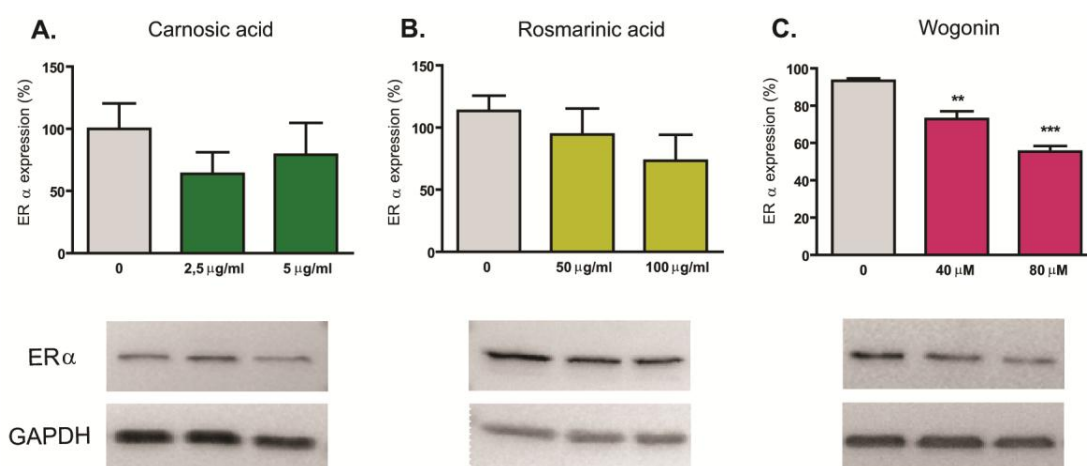


Figure 5. Effects of carnosic acid, rosmarinic acid and wogonin on oestrogen receptor alpha protein expression in T-HESC cells. Western blot analysis was performed on cell homogenates from T-HESC cell cultures treated for 48h with (A) carnosic acid (0; 2.5; 5 µg/ml; $n = 5$); (B) rosmarinic acid (0; 50; 100 µg/ml; $n = 6$); and (C) wogonin (0; 40; 80 µM; $n=5$). The upper panels show quantification of results expressed as a percentage of basal values \pm SEM. Representative blots are presented in lower panels. ** $P < 0.01$; *** $P < 0.001$ versus basal).

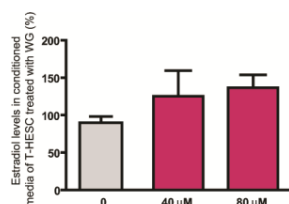


Figure 6. Effects of wogonin on oestradiol levels in conditioned media of T-HESC cells treated with wogonin 40 and 80 µM or vehicle. Enzyme immunoassay was carried out in duplicate for each sample ($n = 5$); bars represent media + SEM. Values are expressed as percentage of vehicle control oestradiol levels set as 100%.

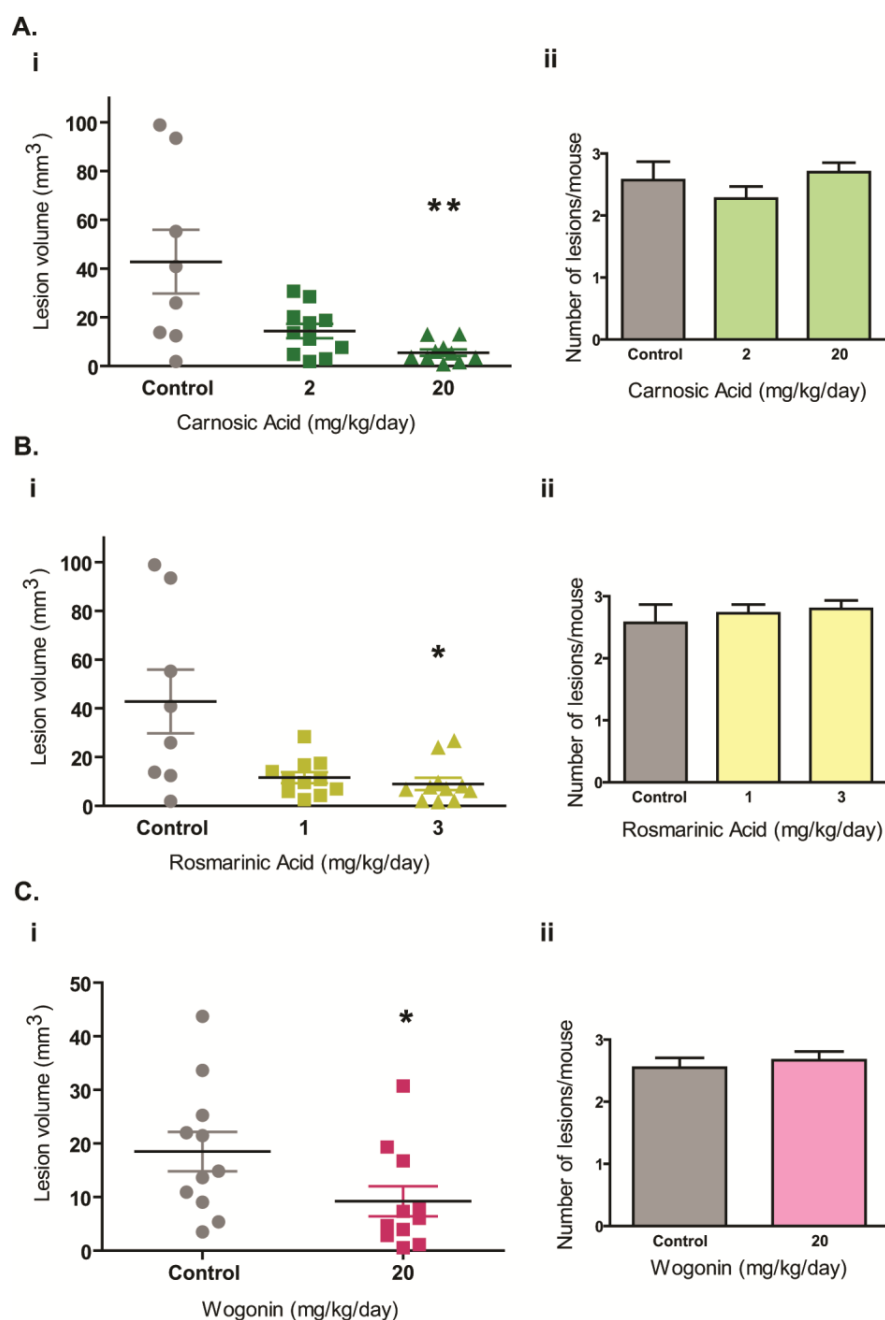


Figure 7. The effect of carnosic acid, rosmarinic acid and wogonin on volume (i) and number (ii) of endometriotic-like lesions. Mice with surgically induced endometriosis were treated with: (A) carnosic acid: 2 mg/kg/day ($n = 11$) and 20 mg/kg/day ($n = 10$) or vehicle (control; $n = 8$); (B) rosmarinic acid: 1 mg/kg/day ($n = 11$) and 3 mg/kg/day ($n = 10$) or vehicle (control; $n = 8$); (C)

wogonin: 20 mg/kg/day ($n = 12$) or vehicle (control, $n = 11$). $*P < 0.05$; $**P < 0.01$ versus control.

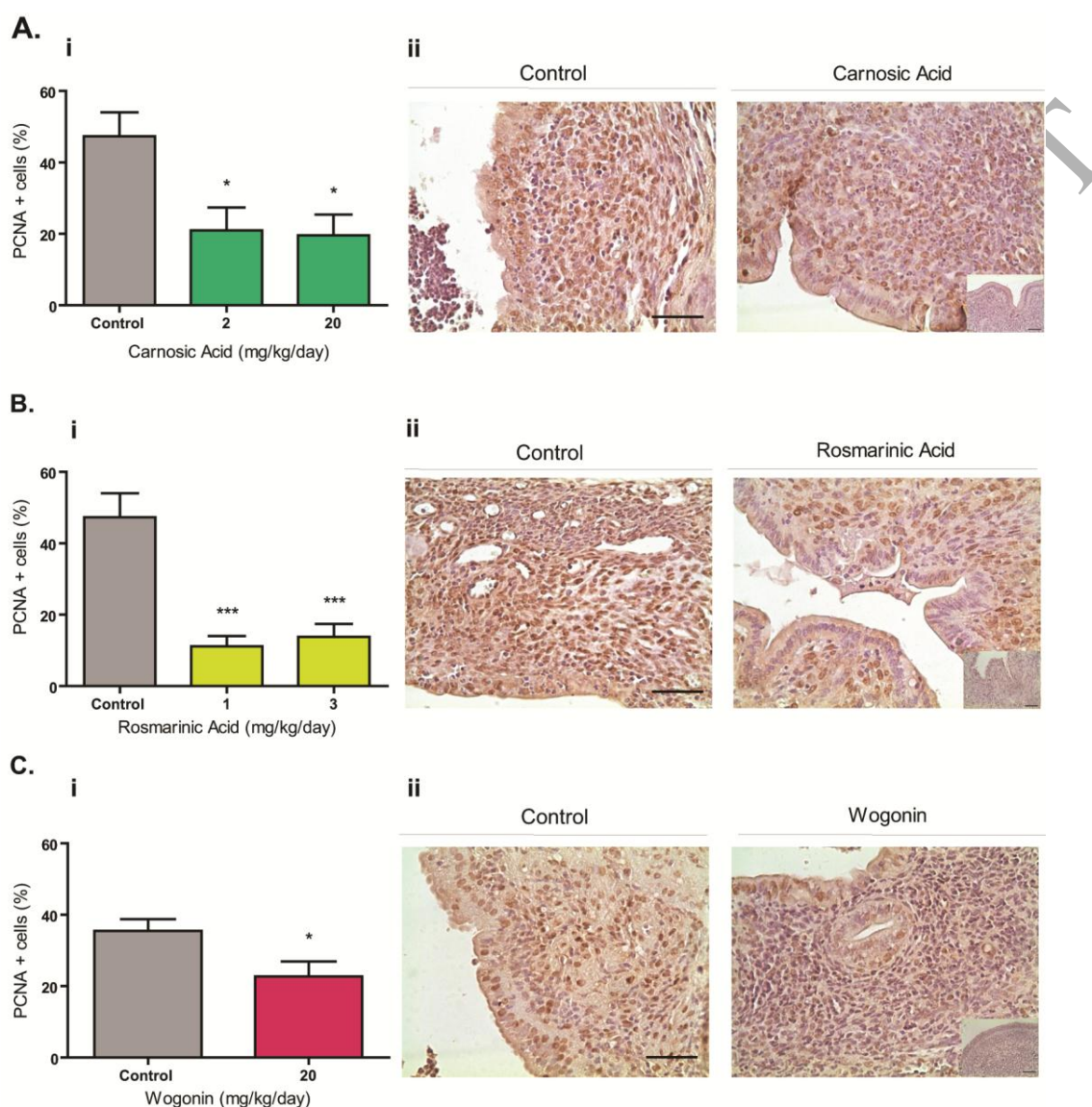


Figure 8. The effect of carnosic acid, rosmarinic acid and wogonin on endometriotic-like lesion cell proliferation. Mice with surgically induced endometriosis were treated with: (A) carnosic acid: 2 mg/kg/day ($n = 11$) and 20 mg/kg/day ($n = 10$) or vehicle (control; $n = 8$); (B) rosmarinic acid: 1 mg/kg/day ($n = 11$) and 3 mg/kg/day ($n = 10$) or vehicle (control; $n = 8$); (C) wogonin: 20

mg/kg/day ($n = 12$) or vehicle (control, $n = 11$). Cell proliferation was evaluated by immunohistochemistry for proliferating cell nuclear antigen (PCNA) in endometriotic-like lesions and was quantified as a percentage of PCNA positive cells (Ai–Ci). $*P < 0.05$; $**P < 0.001$ versus control (Aii–Cii). Photomicrographs of PCNA immunostaining are displayed. Inset: one section of each slide was incubated with a rabbit immunoglobulin G isotype antibody as a negative control. Scale bar: 50 μm . Magnification: 400 X.

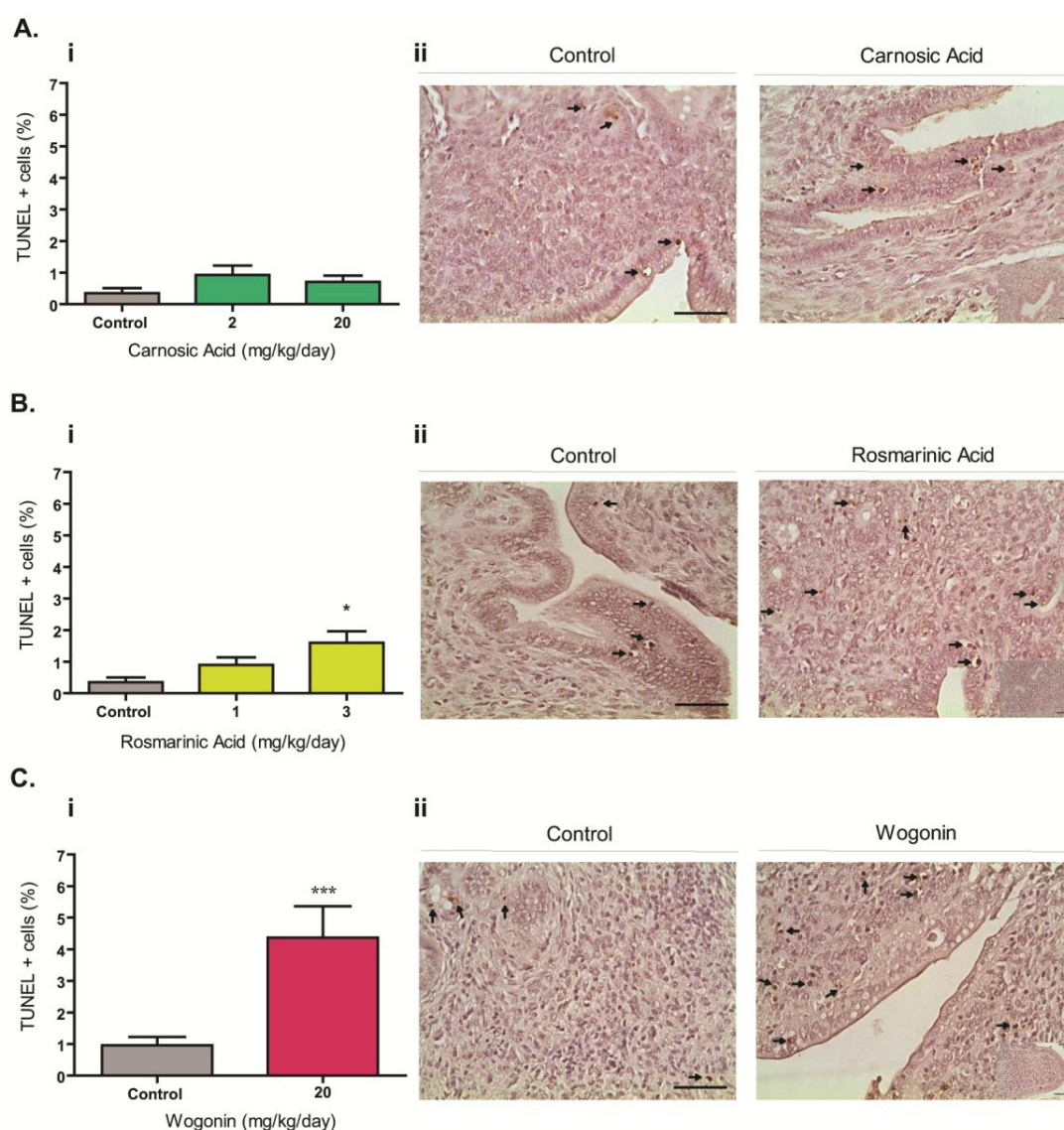


Figure 9. The effect of (A) carnosic acid, (B) rosmarinic acid and (C) wogonin on endometriotic-like lesions apoptosis. Mice with surgically induced endometriosis were treated with carnosic acid: 2 mg/kg/day ($n = 11$) and 20 mg/kg/day ($n = 10$) or vehicle (control: $n = 8$); rosmarinic acid: 1 mg/kg/day ($n = 11$) and 3 mg/kg/day ($n = 10$) or vehicle (control: $n = 8$); wogonin: 20 mg/kg/day ($n = 12$) or vehicle (control, $n = 11$). Apoptosis was assessed by TdT (terminal deoxynucleotidyl transferase) mediated dUDP nick-end labelling (TUNEL) technique and was quantified in endometriotic-like lesions as a percentage of TUNEL positive cells (Ai–Ci). * $P < 0.05$; ** $P < 0.001$ versus control.

(Aii–Cii) Photomicrographs of TUNEL staining are displayed. Black arrows indicate TUNEL positive cells. Inset: negative controls were incubated in the absence of TdT. Scale bar: 50 μ m.

Magnification: 400 X.







Experimental models	Carnosic acid 	Rosmarinic acid 	Wogonin 
Primary cultures 	↓ Cell proliferation inhibition	↓ Cell proliferation inhibition	↓ Cell proliferation inhibition
T-HESC 	↓ Cell proliferation inhibition ✓ G2/M arrest	↓ Cell proliferation inhibition ✓ Antioxidant effect	↓ Cell proliferation inhibition ✓ G2/M arrest ✓ Prooxidant effect ↓ ER α expression
BALB/c 	↓ Decreased lesion volume ↓ Cell proliferation inhibition	↓ Decreased lesion volume ↓ Cell proliferation inhibition ↑ Apoptosis induction	↓ Decreased lesion volume ↓ Cell proliferation inhibition ↑ Apoptosis induction

Figure 10. Effects of carnosic acid, rosmarinic acid and wogonin on the growth of human endometrial stromal cells and on the development of endometriotic-like lesions in a rodent model.

Table 1. Treatment groups, dose and routes of administration.

<i>Treatment groups</i>	<i>Dosage</i>	<i>Duration, days</i>	<i>Route of administration</i>
Control	Vehicle (<i>n</i> = 8)	14	Intraperitoneal injection
	Vehicle (<i>n</i> = 11)	12	Intragastric gavage
Rosmarinic acid	1 mg/kg/day (<i>n</i> = 11)	14	Intraperitoneal injection
	3 mg/kg/day (<i>n</i> = 10)		
Carnosic acid	2 mg/kg/day (<i>n</i> = 11)	14	Intraperitoneal injection
	20 mg/kg/day (<i>n</i> = 10)		
Wogonin	20 mg/kg/day (<i>n</i> = 12)	12	Intragastric gavage